

Role of macula densa nitric oxide and cGMP in the regulation of tubuloglomerular feedback

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Role of macula densa nitric oxide and cGMP in the regulation of tubuloglomerular feedback.

Background. Previous studies have suggested that nitric oxide (NO) produced within cells of the macula densa (MD) modulates tubuloglomerular feedback (TGF). We tested the hypothesis that NO produced in the MD acts locally as an autacoid to activate soluble guanylate cyclase and cGMP-dependent protein kinase in the MD itself.

Methods. Rabbit afferent arterioles (Af-Arts) and attached MD were simultaneously microperfused in vitro. The TGF response was determined by measuring the Af-Art diameter before and after increasing NaCl in the MD perfusate (from 17 mmol/L of Na and 2 of Cl to 65 mmol/L of Na and 50 of Cl). TGF was studied before (control TGF) and after inhibiting components of the NO-cGMP-dependent cascade in the tubular or vascular compartment.

Results. Increasing NaCl concentration in the MD perfusate decreased the Af-Art diameter by $3.2 \pm 0.5 \mu\text{m}$ (from 18.5 ± 1.3 to $15.4 \pm 1.3 \mu\text{m}$, $P < 0.001$). Adding a soluble guanylate cyclase inhibitor (LY83583) to the MD increased TGF response to $6.3 \pm 1.1 \mu\text{m}$ ($P < 0.031$ vs. control TGF). Similarly, when cGMP-dependent protein kinase was inhibited with KT5823, TGF was augmented from 2.6 ± 0.3 to $4.0 \pm 0.7 \mu\text{m}$ ($P < 0.023$). An analogue of cGMP in the MD reversed the TGF-potentiating effect of both 7-nitroindazole (7NI; an nNOS inhibitor) and LY83583. Inhibition of MD guanylate cyclase did not alter the effect of acetylcholine (a NO-cGMP-dependent vasodilator) on the Af-Art. Perfusing the Af-Art with the guanylate cyclase inhibitor did not potentiate TGF, suggesting that the effect of NO occurred at the MD via a cGMP-dependent mechanism. To determine whether the effect of NO in the MD was entirely mediated by cGMP, TGF was studied after giving (1) LY83583 or (2) LY83583 plus 7NI. Adding the nNOS inhibitor to the MD did not potentiate the TGF response further.

Conclusions. We concluded the following: (1) NO produced by the MD inhibits TGF via stimulation of soluble guanylate cyclase, generating cGMP and activating cGMP-dependent protein kinase; (2) NO acts on the MD itself rather than by diffusing to the Af-Art; and (3) most, if not all, of the effect of NO in the MD is due to a cGMP-dependent mechanism rather than to other NO mediators.

Key words: renal function, neuronal NOS, kidney microcirculation, guanylate cyclase, afferent arteriole, vasodilation, autacoid.

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Tubuloglomerular feedback (TGF) is a process by which the macula densa (MD) senses increases or decreases in distal NaCl delivery and causes an increase or decrease in afferent arteriole (Af-Art) resistance, respectively. Thus, TGF participates in the regulation of glomerular filtration rate and distal NaCl delivery [1, 2]. Although the mediators of TGF are not well identified, nitric oxide (NO) produced by the MD modulates the Af-Art vasoconstrictor effect of TGF [3, 4].

Studies both in vivo and in vitro have suggested that NO produced by neuronal NO synthase (nNOS) in the MD partially inhibits TGF [3–5]. However, it is not known whether NO produced by the MD acts as an autacoid on the MD itself or diffuses to the Af-Art, where it causes vasodilation directly.

Nitric oxide has been shown to act via a variety of second-messenger cascades, although most of its effects are mediated by cGMP [6]. NO stimulates generation of cGMP not only in the vasculature but also in the renal tubules, including the proximal tubule, thick ascending limb, and collecting duct [7–10]. However, whether NO inhibits TGF via cGMP or some other mechanism has not been studied.

Here we investigated the following hypotheses: (1) NO produced in the MD by nNOS inhibits TGF via activation of guanylate cyclase, which generates cGMP; this in turn activates a cGMP-dependent protein kinase. (2) Most, if not all, of the effect of NO at the MD is mediated by a cGMP-dependent mechanism, rather than by other mediators; and (3) NO acts as an autacoid at the level of the MD itself, rather than by diffusing to the Af-Art. To test these hypotheses, microdissected rabbit Af-Arts with the MD attached were microperfused in vitro. The TGF response was determined by measuring the Af-Art diameter before and after increasing NaCl in the MD perfusate. TGF was studied before (control TGF) and after inhibiting components of the cGMP cascade in the tubular or vascular compartment. We also studied whether an analogue of cGMP reverses the TGF-potentiating effects of nNOS or guanylate cyclase inhibitors. To determine that drugs perfused into the tubular

compartment did not act in the vascular compartment or vice versa, we studied (1) the Af-Art vasodilator response to acetylcholine (a NO-cGMP-dependent vasodilator) when MD guanylate cyclase was inhibited and (2) whether adding a guanylate cyclase inhibitor to the Af-Art perfusate would potentiate TGF. We found the following: (1) NO produced by the MD inhibits TGF via stimulation of soluble guanylate cyclase, generating cGMP and activating cGMP-dependent protein kinase; (2) NO acts on the MD itself rather than by diffusing to the Af-Art; (3) most, if not all, of the effect of NO in the MD is due to a cGMP-dependent mechanism rather than to other NO mediators; and (4) drugs perfused into the tubular compartment do not act in the vascular compartment and vice versa.

METHODS

Isolation and microperfusion of Af-Arts with MD attached were carried out as described previously [4]. Briefly, young male New Zealand white rabbits were given tap water ad libitum and fed standard rabbit chow; they were then anesthetized with ketamine [50 mg/kg, intramuscularly (IM)], xylazine (50 mg/kg IM), and pentobarbital (40 mg/kg IM) and were given an intravenous injection of heparin (500 U). The kidneys were removed and sliced along the corticomedullary axis, and slices were placed in ice-cold minimum essential medium (MEM; Gibco, Grand Island, NY, USA) containing 5% bovine serum albumin (BSA; Sigma, St. Louis, MO, USA). Using a stereomicroscope (Olympus SZH, Tokyo, Japan) as described previously [4], a single superficial Af-Art and its intact glomerulus from each rabbit were microdissected together with adherent tubular segments consisting of portions of the thick ascending limb, MD, and early distal tubule. The microdissected sample was transferred to a temperature-regulated chamber mounted on an inverted microscope (Olympus IMT-2) with Hoffmann modulation. Both the Af-Art and the end of either the distal tubule or thick ascending limb were cannulated with an array of glass pipettes as described previously [4]. Intraluminal pressure of the Af-Art was measured by Landis's technique, using a fine pipette introduced into the lumen through the perfusion pipette. The Af-Art was perfused with oxygenated MEM (95% O₂; 5% CO₂) containing 5% BSA, and intraluminal pressure was maintained at 60 mm Hg throughout the experiment. The MD was perfused with a modified Krebs-Ringer bicarbonate buffer. The low-NaCl solution contained (in mmol/L) 15 NaHCO₃, 0.96 NaH₂PO₄, 0.24 Na₂HPO₄, 5 KHCO₃, 1.2 MgSO₄, 1.0 CaCl₂, 5.5 glucose, and 1.0 Na acetate; thus, the final Na and Cl concentrations were 17.4 mmol/L Na and 2 mmol/L Cl. The high-NaCl solution had a similar composition except that 48 mmol/L NaCl was added; thus, the final Na and Cl concentrations

were 65.4 mmol/L Na and 50 mmol/L Cl. The MD was perfused at a rate of 10 nL/min.

Bath volume was 1 mL. The bath consisted of MEM containing 0.15% BSA and was exchanged continuously at a rate of 1 mL/min. Microdissection and cannulation were completed within 90 minutes at 8°C, after which the bath was gradually warmed to 37°C for the rest of the experiment. Once the temperature was stable, a 30-minute equilibration period was allowed before taking any measurements. Images were displayed at magnifications of up to $\times 1980$ and recorded with a video system. Af-Art diameter was measured with a MetaMorph image analysis system (Universal Imaging, West Chester, PA, USA).

LY83583, a guanylate cyclase inhibitor, was obtained from Biomol Research Laboratories (Plymouth Meeting, PA, USA); KT5823, a cGMP-dependent protein kinase inhibitor, was obtained from Kamiya Biomedical Co. (Thousand Oaks, CA, USA), and dibutyl- α -methyl-3-isobutylxanthine (DB-MIX) was purchased from Sigma Chemical Co.

Statistics

Each separate experiment was approached in an analogous manner. The designs were repeated measures with the same Af-Art being measured under different conditions. In all cases, a subset of all possible contrasts was specified in advance as being of primary importance. To analyze a specific difference, a paired *t*-test was used. All tests were two-sided, and results are presented as mean \pm SEM. For each experiment, comparisons of interest were considered a set, and Holm's multiple comparisons procedure was used to adjust for multiple testing. *P* < 0.05 was considered significant.

RESULTS

Because NO stimulates cGMP production in other nephron segments via soluble guanylate cyclase, we investigated the effect of adding an inhibitor of soluble guanylate cyclase to either the MD or Af-Art perfusate on TGF as a means of elucidating the mechanism and site of action of NO. First, we studied the effect of the soluble guanylate cyclase inhibitor LY83583 (10⁻⁶ mol/L) on TGF when added to the MD perfusate. When the MD perfusate was changed from low to high NaCl during the control period, the Af-Art diameter decreased from 18.5 ± 1.3 to 15.4 ± 1.3 μ m, a reduction of 3.2 ± 0.5 μ m (*N* = 6). When LY83583 was present in the MD perfusate, changing NaCl from low to high caused the diameter of the Af-Art to decrease from 18.7 ± 1.3 to 12.3 ± 1.5 μ m, a reduction of 6.3 ± 1.1 μ m (*P* < 0.031 compared with control TGF). Thus, TGF was significantly enhanced by inhibiting soluble guanylate cyclase in the MD (Fig. 1).

In a separate series of experiments, we studied

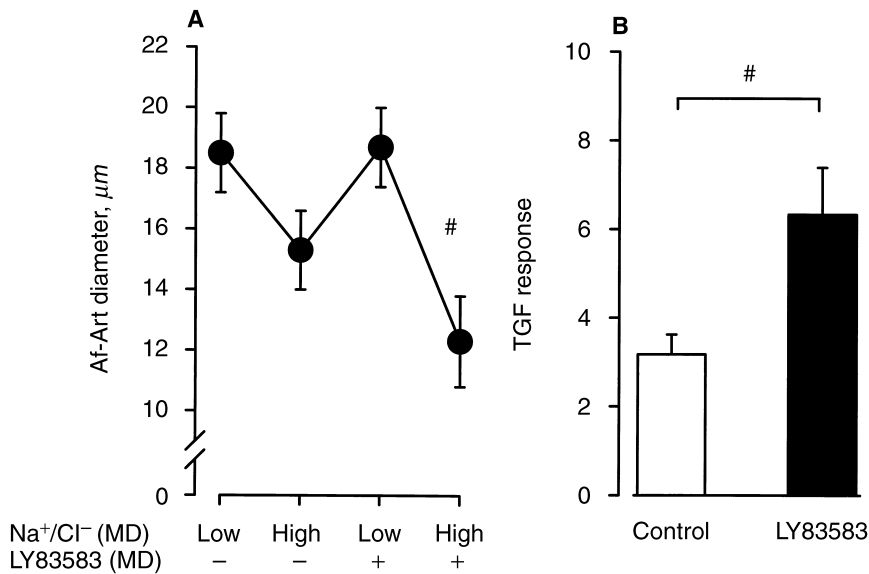


Fig. 1. Effect of LY83583 (10^{-6} mol/L), a soluble guanylate cyclase inhibitor, on the tubuloglomerular feedback (TGF) response to high NaCl [seen as a decrease in the afferent arteriole (Af-Art) diameter]. (A) The macula densa (MD) was perfused with a modified Krebs-Ringer bicarbonate buffer containing either low or high NaCl ($N = 6$). When LY83583 was added to the perfusate during low NaCl perfusion, the diameter did not change; however, during high-NaCl perfusion, LY83583 potentiated TGF. (B) Symbols show the TGF response: (□) control TGF; (■) TGF when LY83583 was added to the MD. [#] $P = 0.031$, changes in control TGF response vs. changes in TGF response when LY83583 was added to the MD.

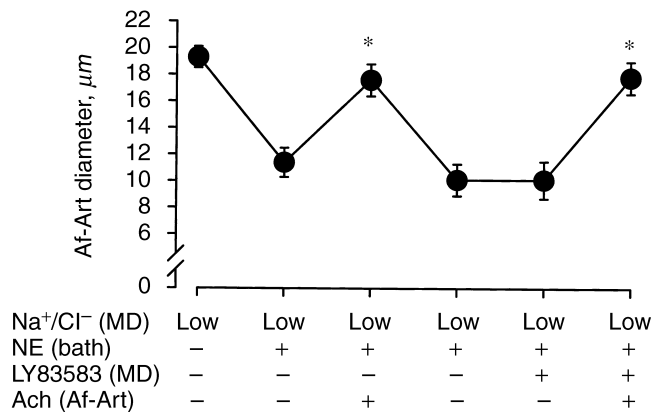


Fig. 2. Vasodilator effect of acetylcholine (ACh) on precontracted Af-Arts, both before and during the addition of the soluble guanylate cyclase inhibitor LY83583 to the MD perfusate. Af-Arts were precontracted by adding norepinephrine (NE) to the bath. When acetylcholine was added to the Af-Art perfusate, diameter increased significantly ($*P = 0.006$); however, the changes caused by acetylcholine in the absence or presence of LY83583 in the MD were the same. Abbreviations are: MD, perfused into the macula densa; bath, superfused into the bath; Af-Art, perfused into the afferent arteriole.

whether the guanylate cyclase inhibitor LY83583 perfused into the MD inhibited the vasodilator effect of acetylcholine on precontracted Af-Arts. When the MD was perfused with low-NaCl solution, adding 2×10^{-7} mol/L norepinephrine to the bath constricted Af-Arts to 59% of basal diameter (Fig. 2). Adding acetylcholine (10^{-6} mol/L) to the arteriolar perfusate increased diameter from 11.4 ± 1.1 to 17.6 ± 1.2 μm ($P = 0.006$). When it was removed, diameter returned to 10.1 ± 1.2 μm . Adding LY83583 to the low-NaCl MD perfusate did not affect diameter. When acetylcholine was now added to the arteriolar perfusate in the presence of LY83583, it

increased diameter from 10.1 ± 1.4 to 17.8 ± 1.2 μm ($P = 0.006$); this increase was similar to that observed in the absence of LY83583. These data indicate that inhibiting MD soluble guanylate cyclase does not affect NO-cGMP-induced dilation of the Af-Art.

We also measured the change in diameter induced by high NaCl before and during perfusion of the Af-Art with LY83583. When the MD perfusate was changed from low to high NaCl during the control period, the Af-Art diameter decreased by 3.0 ± 0.9 μm ($N = 6$; Fig. 3). After adding 10^{-6} mol/L LY83583 to the lumen of the Af-Art, the diameter did not change significantly. When the MD perfusate was changed from low to high NaCl in the presence of LY83583 in the Af-Art, the TGF response was not significantly different from control (Fig. 3). However, the concentration of LY83583 we used completely blocked acetylcholine-induced dilation in precontracted Af-Arts (before LY83583, from 6.1 ± 1.6 to 12.8 ± 1.4 μm ; after LY83583, from 5.7 ± 0.4 to 4.4 ± 0.3 μm , $N = 3$). These data indicate that inhibiting Af-Art soluble guanylate cyclase does not affect TGF. When taken all together, the data also indicate that when a substance is perfused into the MD, it does not directly affect the Af-Art and vice versa.

Because inhibition of soluble guanylate cyclase in the MD augmented TGF, we next investigated whether increasing cGMP in the MD with a permeable analogue of cGMP blunts TGF when MD nNOS or soluble guanylate cyclase is inhibited. When the MD perfusate was changed from low to high NaCl, the Af-Art diameter decreased from 20.3 ± 1.0 to 17.3 ± 1.2 μm ($N = 7$, $P = 0.006$; Fig. 4A). Adding 10^{-5} mol/L 7-nitroindazole (7NI) to the high-NaCl solution (to inhibit nNOS) decreased the diameter further to 15.3 ± 1.5 μm ($P = 0.001$). When

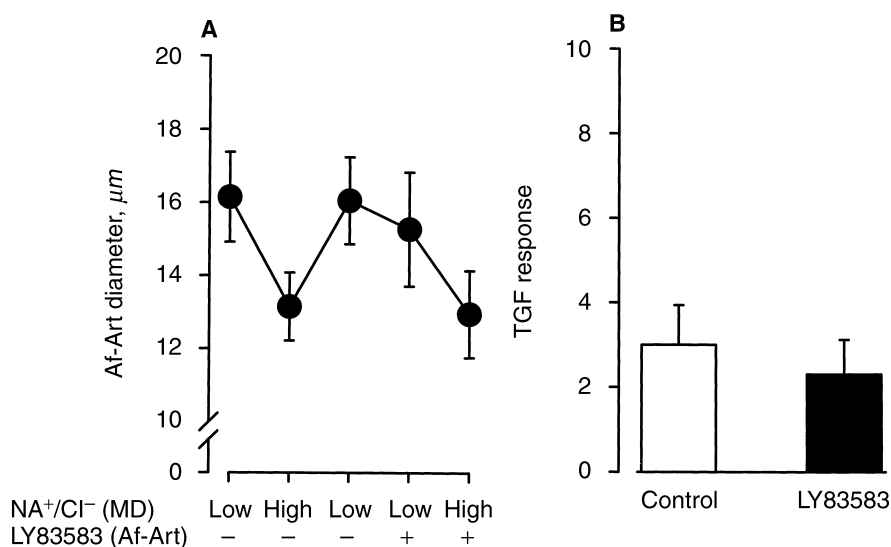


Fig. 3. (A) Changes in Af-Art diameter induced by high NaCl before and during perfusion of the Af-Art with the soluble guanylate cyclase inhibitor LY83583 ($N = 6$). (B) The TGF responses in the absence (□) or presence (■) of LY83583 in the Af-Art were similar. Abbreviations are in the legend to Figure 2.

cGMP was added to the MD perfusate, the Af-Art diameter increased to $18.8 \pm 1.3 \mu\text{m}$ ($P < 0.004$), thus reversing the effect of 7NI. Similar results were found when MD soluble guanylate cyclase was inhibited. When the MD perfusate was changed from low to high NaCl, the Af-Art diameter decreased from 18.2 ± 1.0 to $15.0 \pm 1.1 \mu\text{m}$ ($N = 6$, $P < 0.001$). Adding LY83583 (10^{-6} mol/L) further decreased the diameter from 15.0 ± 1.1 to $12.0 \pm 1.3 \mu\text{m}$ ($P = 0.001$). When cGMP was added to the MD perfusate, the Af-Art diameter increased to $16.7 \pm 1.6 \mu\text{m}$ ($P < 0.002$), reversing the effect of LY83583 (Fig. 4B).

To rule out the possibility that changes in Af-Art diameter reflect a direct effect of cGMP on the arteriole rather than the MD cells, we tested whether adding cGMP to the low-NaCl MD perfusate could dilate the arteriole. When the MD was perfused with low-NaCl solution and 2×10^{-7} mol/L norepinephrine was added to the bath, the Af-Art was reduced to 52% of its original diameter ($P = 0.005$, $N = 5$; Fig. 5). When cGMP was added only to the lumen of the MD while perfusing it with low-NaCl solution, the Af-Art diameter remained unchanged. Subsequently, when acetylcholine (10^{-6} mol/L) was added to the lumen of the Af-Art, the diameter increased from 8.26 ± 1.0 to $15.3 \pm 1.9 \mu\text{m}$ ($P = 0.005$). The data shown in Figures 4 and 5 indicate that directly augmenting MD cGMP can blunt TGF and that cGMP perfused into the MD does not diffuse to the Af-Art in sufficient quantities to alter its diameter (Fig. 5).

cGMP may activate several enzymes, including cGMP-dependent protein kinase. To test whether activation of cGMP-dependent protein kinase is a necessary step in the NO second-messenger cascade, we investigated the effect of the cGMP-dependent protein kinase inhibitor KT5823 (2×10^{-6} mol/L) on TGF. During the control period, when the MD perfusate was changed from low

to high NaCl, the Af-Art diameter decreased from 18.4 ± 1.1 to $15.8 \pm 1.1 \mu\text{m}$, a reduction of $2.6 \pm 0.3 \mu\text{m}$ ($N = 7$). When the MD perfusate was changed from low to high NaCl in the presence of KT5823, the Af-Art diameter decreased from 17.9 ± 1.0 to $13.6 \pm 1.5 \mu\text{m}$, a reduction of 4.0 ± 0.7 ($P < 0.023$ vs. control TGF; Fig. 6). Thus, inhibiting cGMP-dependent kinase in the MD augmented TGF.

Because NO generated at the MD may blunt TGF by activating other second-messenger cascades besides those dependent on cGMP, we investigated the effect of 7NI on TGF while inhibiting MD soluble guanylate cyclase. When the MD perfusate was changed from low to high NaCl in the presence of LY83583, Af-Art diameter decreased by $3.8 \pm 0.8 \mu\text{m}$ (from 19.2 ± 1.5 to $15.4 \pm 1.3 \mu\text{m}$, $N = 6$). Adding 7NI to the MD perfusate in the presence of LY83583 did not constrict the Af-Art further (Fig. 7). In the presence of both inhibitors, when the MD perfusate was switched from low to high NaCl, the diameter decreased by $4.2 \pm 1.6 \mu\text{m}$, not significantly different from control (from 19.6 ± 1.9 to $15.4 \pm 1.3 \mu\text{m}$). Thus, the effect of inhibiting MD soluble guanylate cyclase on TGF was not additive with inhibiting nNOS.

DISCUSSION

Several studies have shown that NO generated by the MD blunts TGF [3–5]. NO produced by the MD could have several sites of action. First, it could act as an autacoid, affecting processes in the MD itself. Alternatively, it could act in the juxtaglomerular mesangial cells, which are most likely vital to transmission of the signal from the MD to the Af-Art. Finally, NO may diffuse from the MD to the Af-Art and thus cause it to dilate. To clarify NO's site of action, we first needed to show where soluble

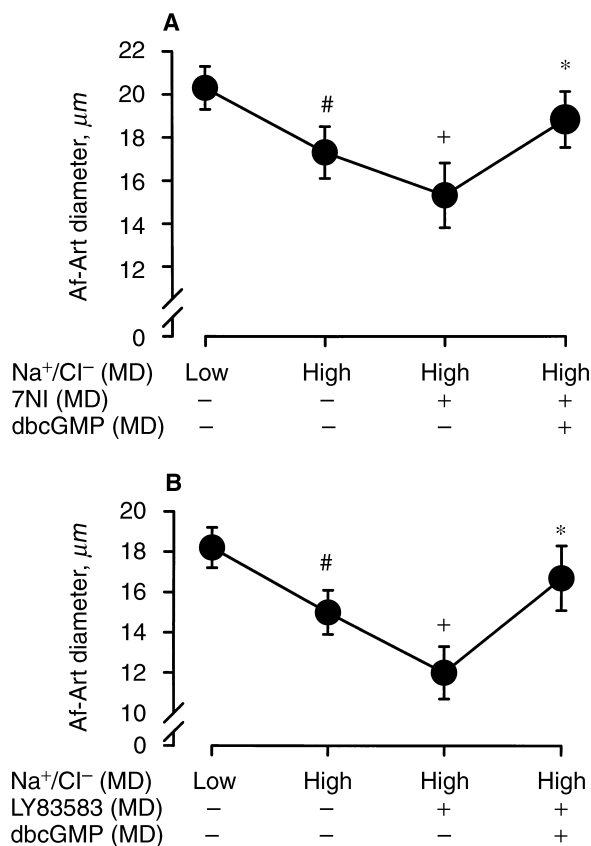


Fig. 4. Effect of dibutyryl cGMP (dbcGMP) on Af-Art diameter when added to the MD perfusate along with high NaCl in the presence of 7NI (A; $N = 7$) or LY83583 (B; $N = 6$). Both 7NI and LY83583 potentiated TGF response. Adding dbcGMP to the MD reversed this effect. $\#P < 0.001$, low vs. high NaCl; $+P < 0.001$, high NaCl vs. high NaCl plus 7NI or LY83583; $*P < 0.004$, 7NI or LY83583 vs. 7NI or LY83583 plus dbcGMP. MD, perfused into the macula densa.

guanylate cyclase is activated by NO produced in the MD. Our data show that adding a soluble guanylate cyclase inhibitor to the tubular perfusate to block it in the MD augments TGF; thus, NO produced in the MD blunts TGF by acting as an autacoid within this specialized plaque of cells rather than by diffusing to the Af-Art and promoting vasodilation. These results cannot be explained by the inhibitor diffusing to the Af-Art or mesangial cells in sufficient quantities to alter NO-induced vasodilation, because (1) directly blunting soluble guanylate cyclase in the Af-Art and mesangial cells by adding LY83583 to the arteriolar perfusate had no effect on TGF, and (2) perfusing the MD with an inhibitor of soluble guanylate cyclase did not alter endothelium-dependent relaxation of the arteriole by acetylcholine. Furthermore, perfusing the MD with dibutyryl cGMP to augment cGMP levels in this segment blunted TGF, but did not dilate Af-Arts precontracted by norepinephrine. Taken together, these data show that NO produced by the MD blunts TGF by acting as an autacoid

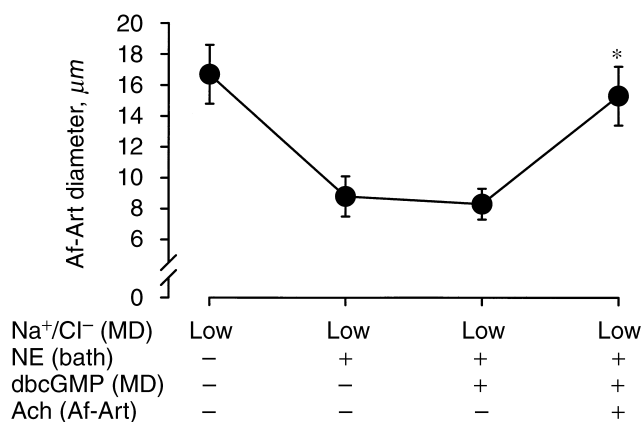


Fig. 5. Effect of dibutyryl cGMP (dbcGMP) on the Af-Art diameter when added to the MD perfusate along with low NaCl ($N = 5$). Norepinephrine (NE) was added to the bath, and acetylcholine (Ach) was added to the arteriolar perfusate. When dbcGMP was added to the MD, it did not cause direct dilation of the Af-Art. The response to Ach was also not affected. $*P = 0.006$, Ach vs. NE plus dbcGMP. Abbreviations are in the legend to Figure 2.

within the MD rather than by diffusing to the mesangium or Af-Art. They also indicate that drugs perfused into the tubular compartment do not act in the vascular compartment and vice versa.

Given that vasoconstrictors such as angiotensin augment TGF and vasodilators such as atrial natriuretic factor (ANF) blunt TGF, one could predict that NO produced by the Af-Art would also blunt TGF because it is a vasodilator. However, directly blunting the activity of soluble guanylate cyclase in the Af-Art and mesangial cells by adding LY83583 to the arteriolar perfusate had no effect on TGF. These data suggest that NO produced by the endothelial cells of the Af-Art does not significantly alter TGF.

Although it is well established that NO inhibits TGF, how it does so remains unclear. To address this question, we investigated the role of cGMP, NO's second messenger in many other tissues [7, 9, 11, 12]. Our data show that when soluble guanylate cyclase was blocked at the MD, TGF was enhanced. Furthermore, when we increased cGMP levels in the MD in the presence of 7NI (to inhibit nNOS) or LY83583 (to inhibit guanylate cyclase), the Af-Art diameter increased. Finally, inhibiting cGMP-dependent protein kinase in the MD also enhanced TGF. From these findings, we concluded that NO stimulates soluble guanylate cyclase in the MD and increases cGMP, which subsequently activates cGMP-dependent protein kinase. Activation of cGMP-dependent protein kinase consequently diminishes another process (as yet unknown) that is necessary for full expression of TGF.

Although our data indicate that NO primarily acts via cGMP and cGMP-dependent protein kinase, the experi-

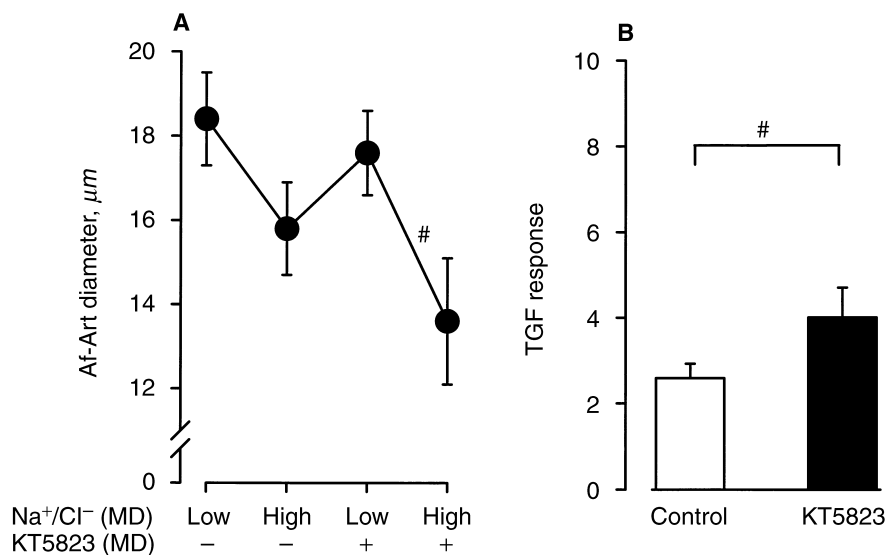


Fig. 6. Effect of the cGMP-dependent protein kinase inhibitor KT5823 (2×10^{-6} mol/L) on TGF response to high NaCl (seen as a decrease in the Af-Art diameter). (A) The MD was perfused with a modified Krebs-Ringer bicarbonate buffer containing either low or high NaCl ($N = 7$). When KT5823 was added to the MD perfusate during low-NaCl perfusion, the diameter did not change; however, during high-NaCl perfusion, KT5823 potentiated TGF. (B) Symbols show the TGF response: (□) control TGF; (■) TGF when KT5823 was added to the MD. $\#P = 0.023$, changes in control TGF response vs. changes in TGF response in the presence of KT5823 in the MD. MD, perfused into the macula densa.

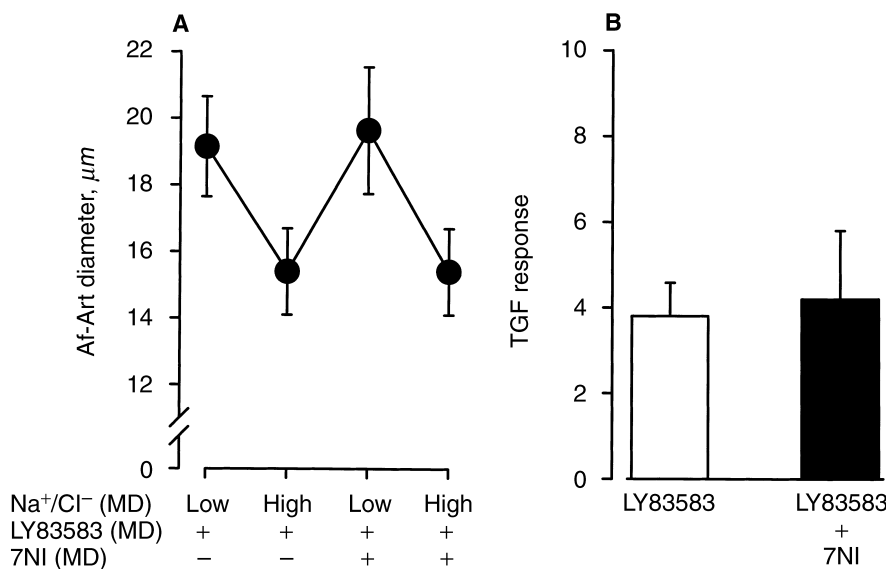


Fig. 7. Effect of the neuronal nitric oxide synthase (nNOS) inhibitor 7NI on TGF response when MD guanylate cyclase was inhibited by simultaneous perfusion of LY83583 ($N = 6$). Symbols are: (□) TGF in the presence of LY83583; (■) TGF with LY83583 plus 7NI. 7NI did not potentiate TGF when MD guanylate cyclase was inhibited. MD, perfused into the macula densa.

ments discussed previously in this article do not rule out the possibility that another second-messenger cascade is activated. To test this possibility, we examined the effect of inhibiting MD nNOS on TGF after MD soluble guanylate cyclase had been inhibited, but found that this had no effect on Af-Art diameter. These data suggest that the effect of NO on the MD is mediated mainly by cGMP.

Several other studies have shown that NO stimulates soluble guanylate cyclase and augments cGMP generation in other tissues [7, 9, 13, 14]. Intravenous infusion of NO donors significantly reduced mean arterial blood pressure and urinary output and increased tubular secretion of cGMP [13]. In the isolated perfused rat kidney, an injection of sodium nitroprusside or acetylcholine

caused a marked release of cGMP, which was reduced by the NOS inhibitor *N*^G-nitro-L-arginine [9]. Inhibiting NOS with *N*^G-monomethyl-L-arginine has been shown to lead to a dose-dependent decrease in cGMP in renal interstitial fluid and urine [7]. In addition, NO alters nephron transport through a cGMP-linked mechanism. Rocznik and Burns showed that sodium nitroprusside (SNP) inhibits Na transport via a cGMP-dependent mechanism in the proximal tubule [10]. We have also shown that NO inhibits transport of water in the cortical collecting duct and Cl in the thick ascending limb by stimulating cGMP production [15, 16].

Increased intracellular cGMP may activate cGMP-dependent protein kinase or affect other cGMP-dependent enzymes [11, 12, 17, 18]. cGMP-dependent protein kinase

has now been implicated in a number of biologically important processes ranging from monitoring intracellular Ca^{2+} levels in smooth muscle cells to regulation of gene expression. In addition, NO inhibits Na,K-ATPase activity in the proximal tubule through a mechanism involving cGMP-dependent protein kinase [12]. We have reported that agents that stimulate cGMP levels in the cortical collecting duct decrease osmotic water permeability via activation of cGMP-dependent protein kinase [15].

In most instances, NO's effects are due to activation of guanylyl cyclase and increases in cGMP synthesis. However, knowledge of NO effects independent of cGMP is growing at a rapid rate. NO metabolites are themselves highly reactive. Peroxynitrite (ONOO^-) can act as a physiological mediator (by activation of cyclooxygenase or other oxidases), or it may have prolonged effects because of nitrosylation of tyrosine residues in proteins [19], interaction with nucleic acids, or induction of apoptosis [20]. Regulation of renin secretion involves different intracellular messengers; renin secretion is inhibited by cGMP [21] and stimulated by cAMP, which appears to be a calcium-related pathway that could involve protein kinase C [22].

Although our data indicate that NO produced by the MD acts as an autacoid via cGMP-dependent protein kinase, they do not address what process in the TGF response is affected. TGF is known to be initiated by transport of Na, K, and Cl by the apical membrane Na/K/2Cl cotransporter. Using isolated perfused thick ascending limbs in vitro, we have shown that NO inhibits NaCl transport, suggesting that locally produced NO acting via an autocrine mechanism may directly affect NaCl transport in the thick ascending limb [16]. In this segment, NaCl absorption is initiated by Na/K/2Cl cotransport. Furthermore, Clemon, Feher, and Baumgarten showed that increased intracellular cGMP induced by ANF inhibits Na/K/2Cl cotransporters in cardiomyocytes [23]. Although most studies indicate that NO has an inhibitory effect, some data suggest that NO stimulates transport in the kidney. In vivo studies and kidney preparations have shown that inhibiting NO results in natriuresis [24], suggesting that NO has an antidiuretic effect. In vitro in the rat medullary thick ascending limb, NO has been found to have a stimulatory effect on HCO_3^- absorption [25], while in the rat proximal tubule it has a biphasic effect [26]. The explanation for the discrepancy regarding the inhibitory effect of NO on transport and the stimulatory effect observed by others is not known. Nevertheless, our results suggest that dynamic NO release during TGF activation may inhibit MD transport, mitigate the TGF response, and serve as an intrinsic and dynamic regulator of the TGF loop, directly counteracting the TGF transmitter.

In conclusion, the present studies indicate that (1) NO generated in the MD blunts TGF by acting within the

MD; (2) NO activates soluble guanylate cyclase, increases cGMP, and stimulates cGMP-dependent protein kinase, which in turn may inhibit transport by the MD; and (3) the cGMP-dependent pathway is the primary second messenger activated by NO.

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